Pharmacogn. Mag.
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ORIGINAL ARTICLE

Toxicogenomics of Phenylhydrazine Induced Hematotoxicity and its Attenuation by Plumbagin from *Plumbago zeylanica*

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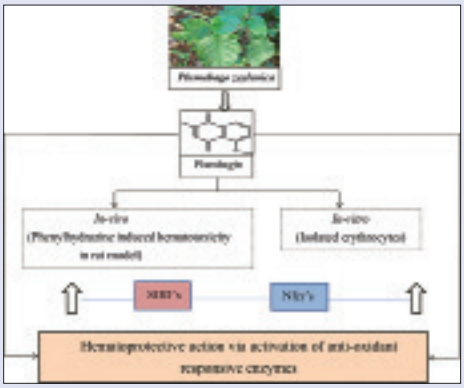
ABSTRACT

Background: High regenerative and proliferative capacity of blood and its components renders it to be at higher risk of adverse drug reactions (ADRs) which are manifested in several treatment regimens against various ailments such as cancers, viral diseases, and several metabolic disorders. **Objective:** It is prudent to come up with some therapeutic entity that can prevent this damage and protects the blood from these ADRs. **Materials and Methods:** We examined protective effects of *Plumbago zeylanica* (PZ) and its active constituent plumbagin (PL) on Sprague Dawley (SD) rats using a phenylhydrazine (Phz) induced hematotoxicity model. Hemoglobin (Hgb), red blood cells (RBCs), mean corpuscular volume, mean corpuscular Hgb (MCH), MCH concentration (MCHC), leukocytes and platelets were studied. Anti-oxidant enzymes superoxide dismutases 2 and 3 (SODs) and nuclear erythroid 2 p45-related factor 1 and 2 (Nfer-1 and 2) were also studied using quantitative real-time polymerase chain reaction (PCR). **Results:** In Phz treated rats, the positive hematotoxic response was obtained in terms of deviated endpoints of blood indices. In PLtreated groups protective response was obtained in terms of normal endpoints of blood indices. In PCR studies, we observed the similar trend. Thus, it can be postulated that PL exerts its protective effects via modulation of anti-oxidant enzymes. **Conclusion:** The study proves that PL can be employed against combatting the ADRs associated with several therapeutic treatment regimens. Similar studies employing such pharmacological entities and their combinations may further prove to be effective against ADRs, especially in the context of blood cells.

Key words: Anti-oxidant enzymes, anti-oxidants, free-radicals, hemato-protective, plumbagin, *Plumbago zeylanica*

SUMMARY

- Hematotoxicity is generally encountered in various therapeutic regimens as ADRs (Adverse Drug Reactions). Plumbagin, an active constituent of plant Plumbago zeylanica is tested for its anti-hematotoxic potential in Phenylhydrazine induced hematotoxicity model in Sprague dawley rats. In vivo, in-vitro and molecular studies confirmed the peremptory actions of PL. It was revealed in our studies that the anti-hematotoxic actions of Plumbagin are due to its capacity to modulate anti-oxidant enzyme system.



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DOI : 10.4103/0973-1296.168983

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Blood and its components are very susceptible toward treatment regimens that are meant for suppressing cell proliferation ex. Chemotherapy, radiation therapy, anti-viral, and anti-epileptic drugs. Such deleterious effects on blood cells and its components are manifested as adverse drug reactions (ADRs). This increased susceptibility of blood toward ADRs arises due to several factors. Blood performs a variety of functions in the body and has a complex biology. Blood cells undergo rapid maturation and proliferation. In a healthy individual, 1–3 million red blood cells (RBCs) are produced each second.[1] Therefore, the drugs and therapies, for example Chemotherapy mend against the proliferative capacity of cells hinder the formation of blood cells and its components. The presence of heme iron makes RBC a potential target for these cancer-killing drugs and therapies as most of them induce heavy oxidative stress for killing cancer cells.[2] Easy occurrence of peroxidation, hemoglobin (Hgb) auto-oxidation, hemolysis, modification in membrane proteins structure, modulation in membrane fragility, these factors make RBC much more susceptible toward oxidative stress and reactive oxygen species (ROS).[3]

Several exogenous and endogenous sources are responsible for induction of ROS in the system. Ultraviolet and other ionizing radiations, environmental pollutants, herbicides, pesticides, and various other chemicals are certain exogenous sources of ROS. Else than these, a weighty amount of ROS is produced endogenously in metabolic processes, especially in incompletely catalyzed reactions.

This ROS is highly detrimental toward blood and its components though a certain degree of ROS is equally required for the system. Various vital functions are performed via ROS including, membrane potential regulation, phosphorylation of bio-molecules, stimulation of cell growth, eicosanoid pathway, and activation of transcription

Cite this article as: Shukla P, Singh RK. Toxicogenomics of Phenylhydrazine Induced Hematotoxicity and its Attenuation by Plumbagin from Plumbago zeylanica. Phcog Mag 2015;11:S380-7.

factors.[4] In theory, there should be a neat counter-balance between free radicals, pro- and anti-oxidants.[5] In cases of increase in ROS, this equilibrium is not attained or is disturbed; the system undergoes stress termed as oxidative stress.[6] This brings oxidative injury to the system leading to secondary manifestations such as RBC membrane degradation, alternations of mitochondrial redox, oxidation of proteins, lipids, and carbohydrates.[7] Oxidative stress is known to be the culprit for various ailments such as cardiovascular diseases, neurological disorders, multiple organ failure, neurodegenerative complications, atherosclerosis, gastro-intestinal disorders, cancer, inflammation, diabetes, and aging.[8]

The attainment of this fine balance between ROS, pro- and anti-oxidants is attributed to various cellular components. Prime of which are anti-oxidant enzyme systems ex. Superoxide dismutases (SODs), catalase, nuclear erythroid 2 p45-related factors (Nfers), glutathione peroxidases (GPx) and peroxiredoxines which include several enzyme with a prime function of checking the increasing ROS.[9]

Taking into account the high amount of oxidative stress posed on blood and hematopoietic system in course of treatment regimens, it is provident to work out some hemato-protective agent that can counter such oxidative stress without hampering the treatment regimen. We focused our study on agents from the natural origin as they have been shown to have enormous potentials with almost nil adverse effects and are used from immemorial times in our traditional medicine systems.[10] PZ exhibits various pharmaceutical properties including antiparkinsonism,[11] anti-malarial,[12] anti-cancer and anti-microbial,[13] anti-inflammatory,[14] anti-genotoxic,[15] hypolipidemic, and anti-atherosclerotic effects activity.[16]

We worked out the traditional claims about the plant PZ being a blood protectant. PZ and its constituents have been shown to have protective actions on lymphocytes,[17] human blood mononuclear cells,[18] erythrocytes[19] and blood coagulation profile.[20] It's positive activity made us to carry out a vigorous survey which stated that the major therapeutic activities imparted by PZ are due to the presence of a naphthoquinone compound, plumbagin (PL).[21] The compound PL exhibits other protective properties which are manifested only due to its anti-oxidant potential such as, protective in spinal cord injury,[22] induces apoptosis in lymphoma cells,[23] anti-inflammatory,[24] anti-diabetic,[25] cell cycle arrest in glioma cells,[26] and anti-tubercular activity.[27]

We used PZ plant ethanolic extract for its protective effects on phenylhydrazine (Phz) induced hematotoxicity model in Sprague-Dawley (SD) rats. In our studies, PZ imparted protective effect on rat blood profile. Similar positive results were obtained against triton-X induced hemolysis in isolated erythrocytes. PL the known isolate active constituent from PZ is supposed to be responsible for these protective effects of PZ. Against Phz induced model of hematotoxicity in SD rats, PL too exhibited protective activity, and concurrent hemato-protective results were obtained in isolate erythrocytes. It was hypothesized that these effects might be attributed to the anti-oxidant activity of the PL. Thus, we performed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay for PL. In DPPH radical scavenging activity, PL came to be a strong anti-oxidant. It was then worked out whether these positive effects are functional at the molecular level. We thus, employed quantitative polymerase chain reaction (q-PCR) approach to work out the mechanism based study of PL on anti-oxidant enzymes, SOD's, and Nfer's.

MATERIALS AND METHODS

Chemicals and kits

Dimethyl sulphoxide (DMSO), 0.9% saline, triton-X, Phz, PL, from *Plumbago zeylnica* (PZ) (PubChem CID: 10205), ethanol, RNAzol were obtained from Sigma-Aldrich, India. Thermo Scientific, India provided SYBR Green and cDNA synthesis kit. Ethylenediamine tetraacetic acid coated vials were procured from Polymedicure Ltd., India. Primers for q-PCR studies were obtained from integrated DNA Technologies.

Ameliorative effects of *Plumbago zeylanica* on complete blood count in rats

Animals and animal care

Total 20 SD albino rats weighing between 120 and 150 g were used in the study. The animals were procured from National Laboratory Animal Facility, CSIR-Central Drug Research Institute (CDRI), Lucknow. Institutional Animal Ethics Committee (IAEC) approval (No. IAEC/2013/70) was taken after submitting details of all the procedures to be performed on animals. Animals were examined routinely for their body weights and weekly for hematological parameters. Venous blood sample was obtained from the rat tail[28] and processed in a fully automatic hematology analyzer. The rats were housed in polypropylene, auto-clavable cages (dimensions: 43 cm × 27 cm × 15 cm) with steel wire-mesh lid having provisions for attaching a water bottle and for keeping food pellets. Animals had permanent access to food and water throughout the study period. They were provided optimized temperature and chronological cycles. At the end of the experiments, all the animals were euthanized following Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) approved Guidelines on animal ethics.

Study design and grouping of animals

Each treatment group comprised of five rats. Group I served as control receiving no treatment at all. Group II, III, and IV were administered with 100, 200 and 400 mg/kg body weight of PZ ethanol extract mixed in distilled water.

Induction of hematotoxicity

Phz is well known to induce hematotoxicity.[29] Phz was administered orally to all groups (except control) at the dose of 8 mg/kg body weight daily for a duration of 7 days. Induction of hematotoxicity was confirmed by complete blood count (CBC). After dosing of Phz for 7 days a significant change was observed in CBC, which confirmed the induction of Hematoxicity in SD rats.

Collection and preparation of Plumbago zeylanica ethanol extract

PZ L. (Leaves) were procured from local areas of Lucknow within the Institute campus. The collection and identification of plant were performed by the permission and project funds of Director-CDRI. The plant was authenticated and verified by matching with herbarium specimens kept in the herbarium collection of Division of Botany, CSIR-CDRI, Lucknow. The leaves were shade dried for 10 days, then grounded to obtain a fine powder. This powder was soaked in ethanol for about 72 h, followed by continuous stirring. This was then filtered. The obtained solvent was completely evaporated under reduced pressure below 50°C to obtain a thick, viscous extract termed as ethanol extract of PZ.

Attenuating effects of Plumbago zeylanica

PZ was administered at a dose of 100, 200, and 400 mg/kg body weight[19] to the test animals as per their respective treatment groups. Blood parameters were observed after 7 days for any deviations from their normal endpoints.

Effect on erythrocytes

Method followed was from Sivonová *et al.*, with slight modifications.[30]

Preparation of solutions

Stock solution (10 mg/ml) of ethanol extract of PZ was prepared by dissolving it in DMSO. This was then diluted up to 5-fold.

Measurement of hemolysis

Hemolysis was observed by spectrophotometric quantification. RBC suspension was centrifuged at 2000 rpm for 10 min, then washed with 0.9% saline thrice. The suspension thus obtained was incubated with plant dilutions for 1 h at 37°C. Absorbance was read at 540 nm using spectrophotometer.

Ameliorative effects of plumbagin on complete blood count in rats

Animals and animal care

As described in section 2.2.1.

Study design and grouping of animals

Each treatment group comprised four rats each. Group I served as control and received no treatment at all. Group II received Phz for 1-week; no treatment in the consecutive week and was termed as a toxin control group. Whereas, Group III was treatment group receiving Phz treatment for 1-week and PL treatment for the successive week.

Induction of hematotoxicity

Phz was administered orally to treatment groups at the dose of 8 mg/kg body weight daily. Significant deviation compared to normal endpoints in CBC was considered as induction of hematotoxicity.

Attenuating effects of plumbagin

PL was administered at a dose of 10 mg/kg body weight[31] to the test animals as per their respective treatment groups. Blood parameters were observed after 7 days for any deviations from their previous values.

Effect on erythrocytes

As described in section 2.3.

Anti-oxidant activity of plumbagin

The procedure described by Grace-Lynn *et al.* 2012[32] was followed with certain modifications. The free radical scavenging activity of PL was determined employing stable DPPH radical. At increasing concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/ml PL was used. The experiment was performed in triplicates. The absorbance was recorded using spectrophotometer at 517 nm. The percentage of DPPH utilized in reaction was recorded using following equation: DPPH scavenging effect (%) = ([A0 – A1]/A0) × 100; where, A0 was the absorbance of PL and A1 was the absorbance in the presence of the methanol (positive control)).

Effect of plumbagin on anti-oxidant enzyme system

RNA isolation and cDNA preparation

RNA isolation was performed by RNAzol method as prescribed by manufacturer's protocol. Total RNA was isolated from blood, checked for quality, purity, and concentration with nanodrop. This quantified RNA was the converted into cDNA using a revert aid first strand cDNA synthesis kit from thermo Scientific as prescribed by manufacturer's protocol. About 5 ng RNA from each group was reverse transcribed with oligo-dT primers. These cDNA templates were again quantified for quality and purity.

Quantitative real-time polymerase chain reaction

Fold change of rat Nfer-1, Nfer-2, SOD-1 and SOD-2 were measured by single-step real-time PCR. 25 ng of total cDNA was taken as a template in each reaction. Rat beta-actin was taken as control.

- Primer sequences
  - SOD-2 Forward Primer: AGCGTGACTTTGGGTCTTT
  - SOD-2 Reverse Primer: AGCGACCTTGCTCCTTATTG
  - SOD-3 Forward Primer: CCTAGAGACCTCGTTTGTCTTC
  - SOD-3 Reverse Primer: TAGGGATGTGTGTGTGTGTG
  - Nfer-1 Forward Primer: GCAGTGGACCTGACTGTATTT
  - Nfer-1 Reverse Primer: CTTTGCCTGTCTGCAGTATCT
  - Nfer-2 Forward Primer: CTTCCTCTGCTGCCATTAGT
  - Nfer-2 Reverse Primer: CAGTGTGCTTCTGGTTGAAAG.
- Primer designing
  - Integrated DNA Technologies Inc., (US) Primer Quest Software.
- Primer source
  - Integrated DNA Technologies Inc., (US).

Statistics

The data generated was subjected to *t*-test for calculating the value of significance (*P* value) using Graph pad prism. Data are expressed as mean ± standard deviation A value of $P \leq 0.05$ was used as a criterion for significance.

RESULTS

Ameliorative effects of *Plumbago zeylanica* on complete blood count in rats

Induction of hematotoxicity

Hematology reports confirmed the induction of hematotoxicity after administration of Phz for duration of 7 days at the dose of 8 mg/kg body weight. This was manifested as significant deviations of endpoints in CBC when compared with the initial values. Initial hematology report showed the normal values of blood indices in control and treated groups. After induction of hematotoxicity, the median hematology report shows significant change in the values of blood parameters with Hgb, RBC, hematocrit (Hct) and mean corpuscular Hgb concentration (MCHC) exhibiting 35%, 60%, 29%, and 25% reduction, respectively. While mean corpuscular volume (MCV), mean corpuscular Hgb (MCH), leukocyte (TLC) and platelets exhibited a significant 36%, 77%, 45%, and 70% enhancement in their values respectively. The counts of subjects from the control group were unaltered in all the CBC reports [Tables 1 and 2].

Attenuating effects of Plumbago zeylanica

Oral ethanol extract of PZ for 1-week induced a significant attenuation of Phz induced hematotoxicity in hemato-compromized rats. This attenuating effect was observed as restored blood indices. The percent increase values of Hgb, RBC, Hct, MCHC were observed to be 55%, 126%, 20%, and 16%, respectively when compared to the median hematology report. The blood parameters which were increased from their normal values in the previous hematology reports were also restored to normal with decrease of about 24%, 38%, 28%, 36% in MCV, MCH, TLC and platelets, respectively [Table 3].

Effects on erythrocytes

PZ dilutions as obtained from section 2.3 were tested for their hematoprotective activities on isolated erythrocytes. At increasing dilutions of 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml and 0.0001 mg/ml, the change compared with positive control was observed to be 18.2, 78.7, 92.7, 93.8, and 94.8, respectively, confirming the hemato-protective activity of PZ ethanol extract on isolated erythrocytes [Figure 1].

Ameliorative effects of plumbagin on complete blood count in rats

Induction of hematotoxicity

Hematotoxicity was induced as described in section 3.1.1. Initial

Table 1: Quantitative analyses of various hematological endpoints at the on-set of experiment. Group I, II, III and IV: Control, PZ treated at 100, 200 and 400 mg/kg body weight respectively. Data is presented as Mean±S.D. values. Statistical significance for Groups II, III and IV compared with respect to Group I (control). (δ=ns)

Groups	Hgb (g/dl)	RBC (X10^{6}µL)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	TLC (X10^{3}µL)	Platelet (X10^{3}µL)
I	11.16±0.59	6.32±0.68	36.74±3.21	62.16±2.86	14.54±1.87	32.82±2.6	15.06±1.11	538.8±71.7
II	11.44±1.05$^\delta$	6.56±0.45$^\delta$	36.54±2.65$^\delta$	62.14±3.26$^\delta$	15.2±1.13$^\delta$	32.86±3.03$^\delta$	15.92±1.53$^\delta$	599.8±78.12$^\delta$
III	11.06±1.18$^\delta$	6.76±0.27$^\delta$	38.94±2.86$^\delta$	63.12±1.51$^\delta$	13.74±1.7$^\delta$	33.44±1.41$^\delta$	14.28±2.04$^\delta$	590.6±147.25$^\delta$
IV	11.8±0.55$^\delta$	6.88±0.48$^\delta$	36.42±2.17$^\delta$	61.46±2.9$^\delta$	14.7±1.29$^\delta$	32.16±2.21$^\delta$	16.6±1.74$^\delta$	482.4±79.28$^\delta$

PZ: *Plumbago zeylanica*; Hgb: Hemoglobin; RBC: Red blood cells; Hct: Hematocrit; MCV: Mean corpuscular volume; MCHC: MCH concentration; TLC: Leukocyte; SD: Standard deviation

Table 2: Quantitative analyses of various hematological endpoints after dosing of Phz at 8 mg/kg body weight for one week to all the groups except control. Group I, II, III and IV: Control, PZ treated at 100, 200 and 400 mg/kg body weight respectively. Data is presented as Mean±S.D. values. Statistical significance for Groups II, III and IV compared with respect to Group I (control). (*P<0.05; **P<0.005, ***P<0.001; δ=ns)

Groups	Hgb (g/dl)	RBC (X10^{6}µL)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	TLC (X10^{3}µL)	Platelet (X10^{3}µL)
I	11.26±0.76	7.04±0.73	40.12±5.33	61.24±1.76	12.64±6.52	28.12±2.5	15.64±3.24	512.6±80.55
II	6.96±1.21***	3.18±0.69***	26.26±3.77**	83.84±4.87***	28.86±3.92**	24.24±1.5*	21.58±2.96*	683.8±46.4**
III	8.08±1.37**	2.6±0.44***	37.28±3.27**	80.3±4.61***	26.62±1.99**	24.08±1.48*	25.74±4.48**	714±70.22**
IV	7.58±1.27***	2.75±0.37***	28.22±4.5**	83.92±2.11***	26.12±2.99**	23.96±1.92*	24.15±2.56**	821.2±155.03**

PZ: *Plumbago zeylanica;* Phz: Phenylhydrazine; Hgb: Hemoglobin; RBC: Red blood cells; Hct: Hematocrit; MCV: Mean corpuscular volume; MCHC: MCH concentration; TLC: Leukocyte; SD: Standard deviation

Table 3: Quantitative analyses of various hematological endpoints after dosing of DIEE for one week to all the groups except control. Group I, II, III and IV: Control, PZ treated at 100, 200 and 400 mg/kg body weight respectively. Data is presented as Mean±S.D. values. Statistical significance for Groups II, III and IV compared with respect to Group I (control). (*P<0.05; **P<0.005, ***P<0.001; δ=ns)

Groups	Hgb (g/dl)	RBC (X10^6 µL)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	TLC (X10^3 µL)	Platelet (X10^3 µL)
I	11.24±0.58	6.82±0.64	37.34±3.51	63.18±1.42	15.82±2.19	28.7±2.19	15.7±3.15	517.2±81.08
II	11.48±1.03$^\delta$	6.42±0.9$^\delta$	32.88±2.54$^\delta$	64.62±1.94$^\delta$	14.58±/2.09$^\delta$	27.6±2.15$^\delta$	17.44±2.19$^\delta$	491.4±87.06$^\delta$
III	11.68±1.28$^\delta$	6.02±0.81$^\delta$	32.92±4.11$^\delta$	63.7±0.98$^\delta$	16.12±1.85$^\delta$	27.26±1.74$^\delta$	15.04±1.35$^\delta$	553.2±77.7$^\delta$
IV	11.78±0.98$^\delta$	6.24±0.67$^\delta$	34.14±1.9$^\delta$	63.7±2.22$^\delta$	16.08±1.39$^\delta$	27.92±1.33$^\delta$	17.26±2.99$^\delta$	522.6±93.4$^\delta$

PZ: *Plumbago zeylanica;* Hgb: Hemoglobin; RBC: Red blood cells; Hct: Hematocrit; MCV: Mean corpuscular volume; MCHC: MCH concentration; TLC: Leukocyte; SD: Standard deviation; DIEE: Dillenia indica ethanolic extract

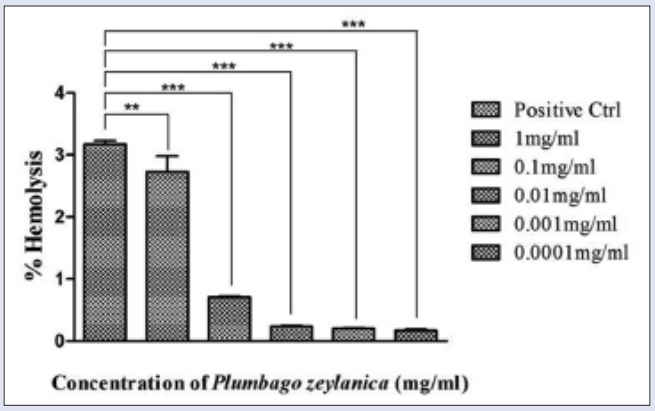

Figure 1: Graphical representation of hematoprotective effect of ethanol extract of *Plumbago zeylanica* on erythrocytes. Hematoprotective effect on erythrocytes increases with the increasing concentration of *Plumbago zeylanica* in mg/ml. Data is presented as mean ± standard deviation values. Statistical significance for all the concentrations of *Plumbago zeylanica* compared with respect to positive control. (** P < 0.005; *** P < 0.001)

hematology report showed normal values of all blood parameters. After 7 days of dosing with Phz, there was a significant reduction in the counts of blood parameters Hgb, RBC, Hct, and MCHC exhibiting 40.8%, 87%, 17%, and 29% reduction, respectively. While, MCV, MCH, TLC and Platelets exhibited a significant 27%, 7%, 9%, and 33% increase, respectively. The counts of subjects from the control group were unaltered in initial hematology report [Table 4].

Attenuating effect of plumbagin

The treatment of hemato-compromised rats with PL for 7 days was observed to induce recovery in altered blood parameters. The restoration of Hgb, RBC, Hct, and MCHC was observed as an increase of 109%, 165%, 31%, and 42% respectively when compared to the hemato-compromised condition. The blood indices which were increased form their normal values in the hemato-compromised condition were also restored to normal with decrease of about 47%, 20%, 2.5%, 33% in MCV, MCH, TLC, and platelets, respectively [Table 5].

Effect on erythrocytes

At increasing dilutions of PL at 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml, and 0.0001 mg/ml, hematoprotective activity was observed. This indicates that PL has positive effects on erythrocytes at varying tested concentrations. At dilution of 1 mg/ml percentage change with respect to positive control was 64.32%. At increasing dilutions of 0.1, 0.01, 0.001, and 0.0001 mg/ml the percentage change with respect to triton-X-100, which was positive control was measured to be 75.40%, 77.92%, 81.08%, and 81.71%, respectively [Figure 2].

Anti-oxidant activity of plumbagin

The stable DPPH radical was scavenged in a dose-dependent manner. With increasing concentrations of PL at 0.5 µg/ml, 1.0 µg/ml, 1.5 µg/ml, 2.0 µg/ml and 2.5 µg/ml, scavenging of DPPH by PL also increased. The result suggests that PL exhibits strong anti-oxidant activity [Figure 3].

Effect of plumbagin on anti-oxidant enzyme system

Effect on superoxide dismutases

The results obtained shows that mRNA expression of SOD 2 and SOD 3

Table 4: Quantitative analyses of various hematological endpoints after one week dosing of Phz to all groups except control. Group I is Control, Group II Toxin control and Group III is Plumbagin treated. Data is presented as Mean±S.D. values. Statistical significance for Groups II and III compared with respect to Group I (Control). (*P<0.05; **P<0.005; ***P<0.001)

Groups	Hgb (g/dl)	RBC (X10^6 µL)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	TLC (X10^3 µL)	Platelet (X10^3 µL)
I	13.1± 1	7.5±1.0	40.4±4.1	55.0±3.3	19.0±2.2	31.8±2.5	14.8±5.4	325.4±113.2
II	5.43±0.7***	2.1±0.3***	26.7±2.8**	122.1±7.9***	25.05±1.44**	20.3±1.0***	23.8±4.7*	741.5±88.58**
III	5.25±0.79***	2.3±0.4***	27.2±3.7**	119.6±15.0***	25.3±0.8**	21.5±1.9***	24.5±4.9*	821.2±125.18**

Phz: Phenylhydrazine; Hgb: Hemoglobin; RBC: Red blood cells; Hct: Hematocrit; MCV: Mean corpuscular volume; MCHC: MCH concentration; TLC: Leukocyte; SD: Standard deviation

Table 5: Quantitative analyses of various hematological endpoints after one week dosing of phz to all groups except control. Group I is Control, Group II Toxin control and Group III is Plumbagin treated. Data is presented as Mean±S.D. values. Statistical significance for Groups II and III compared with respect to Group I (control). (*P<0.05; **P<0.005, ***P<0.001; δ=ns)

Groups	Hgb (g/dl)	RBC (X10^6 µL)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	TLC (X10^3 µL)	Platelet (X10^3 µL)
I	12.7±1.0	7.0±0.6	36.9±3.4	55.9±4.9	18.0±1.0	31±4.41	15.4±5.1	347.48±149.8
II	9.08±1.7***	4.0±0.5***	21.75±4.6**	88.5±4.0***	23.2±1.54**	26.2±1.0***	29.1±5.1*	756.4±128.72**
III	12.15±1.7^δ	6.1±0.7^δ	38.6±5.13^δ	60.85±4.8^δ	18.26±0.72^δ	31.4±1.3^δ	18.8±2.1^δ	502.0±140.45^δ

Phz: Phenylhydrazine; Hgb: Hemoglobin; RBC: Red blood cells; Hct: Hematocrit; MCV: Mean corpuscular volume; MCHC: MCH concentration; TLC: Leukocyte; SD: Standard deviation

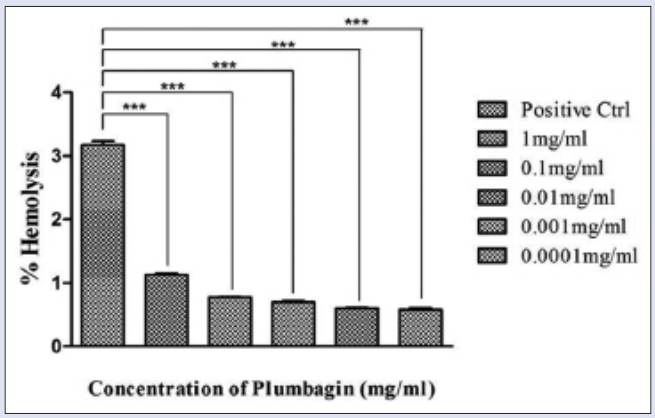

Figure 2: Graphical representation of hematoprotective effect of plumbagin on erythrocytes. Hematoprotective effect on erythrocytes increases with the increasing concentration of plumbagin in mg/ml. Data is presented as mean ± standard deviation values. Statistical significance for all the concentrations of *Plumbago zeylanica* compared with respect to positive control. (*** P < 0.001)

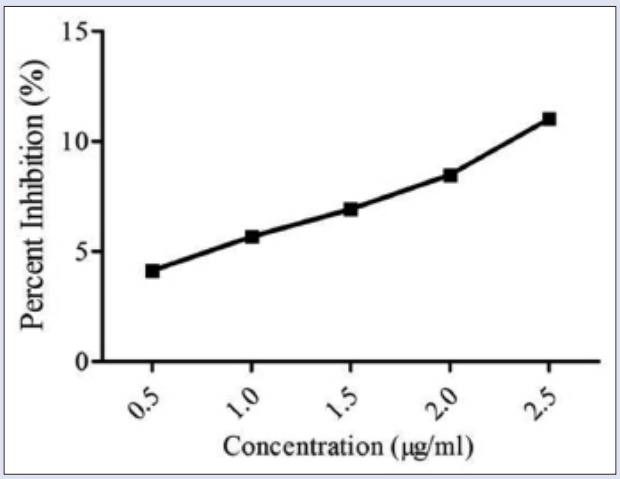

Figure 3: Graphical representation of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of plumbagin at various concentrations. Percentage inhibition of 1,1-diphenyl-2-picrylhydrazyl by plumbagin increases with the increasing concentration of plumbagin in µg/ml. The data is plotted as data is presented as mean ± standard deviation values

were higher in the treatment group receiving PL (T.2) compared to the toxin control (T.1) [Figures 4a and b and 5a and b].

Effect on nuclear erythroid 2 p45-related factors

The quantification of Nfer-1 and 2 gene expression revealed that the m-RNA expression levels of PL treated group (T.2) was significantly higher than the Phz treated groups (T.1) [Figures 5a and b and 6].

DISCUSSION

The main mechanism of Phz induced hematotoxicity is the generation of free radicals, causing damage to the biological system.[33] These free radicals lead to an influx of ROS in a system which leads to disturbed equilibrium between free radicals and anti-oxidants.[34] Blood and its components being very susceptible towards any changes in this equilibrium and thus are affected the most. Unchecked accumulation of ROS leads to various deleterious effects such as hemolysis of erythrocytes mortality of hematopoietic stem cells and their precursors. This finally leads to damaged blood cells and other hematological malignancies including hematotoxicity.[35] These deleterious effects are manifested ADRs in several therapeutic regimens.

The study was designed to come up with some entity which can ameliorate the ROS-mediated ADRs without hingering the treatment regimens of various drugs. The study was focused on the entities with anti-oxidant properties via which hindered equilibrium between ROS and anti-oxidants can be restored. Plants are known to be the abounding sources of natural anti-oxidants.

PZ also known as "chitrak" goes back to early times in traditional medicine. PZ has exhibited anti-oxidant propertiesi various assays. PZ being strong anti-oxidant has shown to have various therapeutic effects.[36] Compounds PL, isoshinanolone, plumbagic acid, beta-sitosterol, 4-hydroxybenzaldehyde, trans-cinnamic acid, vanillic acid, 2, 5-dimethyl-7-hydroxychromone, and indole-3-carboxaldehyde have been isolated.[37]

PL has been isolated and considered as the main active principle for therapeutic properties of PZ. Anti-oxidant potential of PL has been

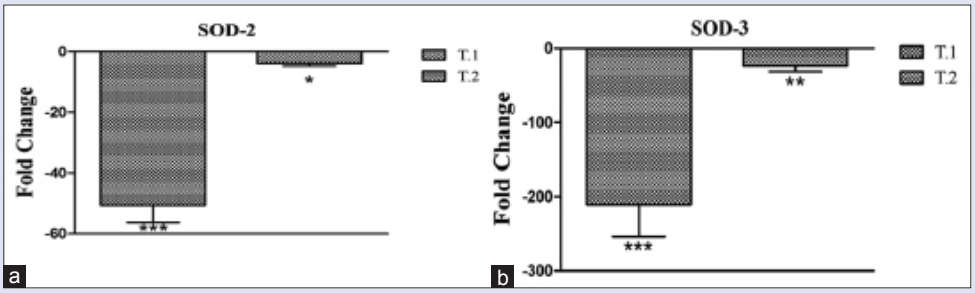

Figure 4: Graphical representation of fold change of mRNA expression levels as observed in quantitative-polymerase chain reaction for superoxide dismutases-2 (a), superoxide dismutases-3 (b). Data presented as mean ± SD; (****P* = 0.001; ***P* = 0.005; **P* = 0.05)

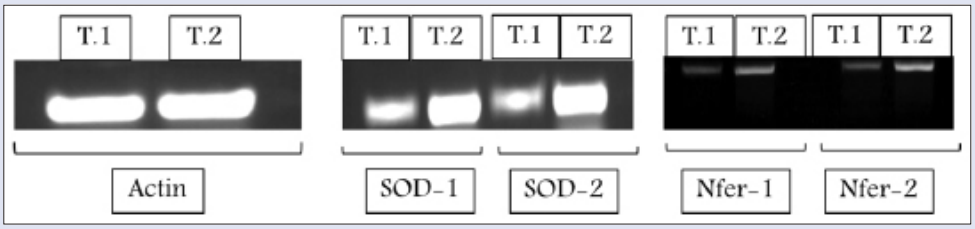

Figure 5: Gel image suggesting up and down regulated enzymes; superoxide dismutases-2, superoxide dismutases-3, nuclear erythroid 2 p45-related factor-1 and nuclear erythroid 2 p45-related factor-2 compared to rat beta-actin control

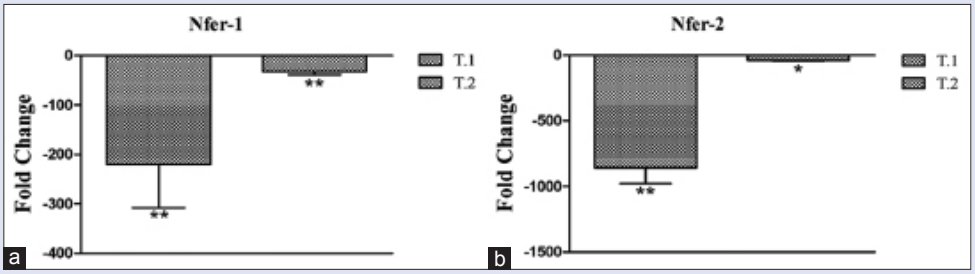

Figure 6: Graphical representation of fold change of mRNA expression levels as observed in quantitative-polymerase chain reaction for nuclear erythroid 2 p45-related factor-1 (a) and nuclear erythroid 2 p45-related factor-2 (b). Data presented as mean ± standard deviation; (***P* = 0.005; **P* = 0.05)

well elucidated.[38] PL acts as pro-oxidant and the therapeutic effects are mediated through lowering of oxidative stress in the biological system.[23]

In a biological system, a fine balance is maintained amongst pro- and anti-oxidants. This role is majorly governed by anti-oxidant enzymes such as: SODs, catalase, glutathione, heme-oxygenase, GPx, Nfer, and NADPH oxidase. They are the first line of defense against exogenously induced oxidative stress, and their prime function is to prevent oxidative injury.[39]

There are three major SODs in the mammalian system. SOD-1 (also known as Cu-Zn SOD), SOD-2 (also known as Mn-SOD) and SOD-3 (also known as extracellular SOD) – amongst which SOD-2 and SOD-3 are known to have of prime importance in hematopoietic system. SODs are metalloproteins that protect organisms from toxic ROS by catalyzing the conversion of superoxide anion to hydrogen peroxide and molecular oxygen.[40] SOD-3 has not been very well understood but is of prime significance in terms of Nfer-2 mediated intrinsic defense against oxidative injury.[41]

Nfer mediates primary protection of cellular machinery from ROS inducing sources via activation of other anti-oxidative enzymes. Nfer has been known to protect cells from exogenous xenobiotics, toxicants, and other deleterious effects by stimulating other anti-oxidant genes.[42] Nfer-2 is a redox-sensitive, basic leucine zipper protein that regulates the transcription of several antioxidant genes and are essential for normal differentiation of erythroid precursors.[43] Nfer-1 and 2 are omnipresent and also have a role to play in the regulation of intrinsic anti-oxidant defense machinery.[44]

The study was focussed on finding out the ameliorative effects of PZ and PL on Phz induced hematotoxicity. The study also revealed the effect of PL on anti-oxidant enzymes SODs and Nfers. Our work reveals that the protective effects of PZ and its active constituent PL are imparted due to its potential to enhance the enzymes involved in the defense against the oxidative stress namely SODs and Nfers. The hemato protective activities of both PZ and PL were elucidated in both *in vitro* and *in vivo* systems. The study reveals that the effects manifested are majorly due to the anti-oxidant potential of PL.

The pro- and anti-oxidant therapeutic nature of PL can be understood as a form of hormesis which states that the low levels of stressors initiates such an adaptive response that is protective in nature.

We evaluated protective effects of PL from PZ and revealed that such natural therapeutic agents can play an important role in overcoming ADRs which are manifested due to various treatment regimens including cancer therapy. Enhancement of Nfer-2 by PL is in itself a novel finding. As Nfer activation is directly co-related with suppression of cancer.[45]

CONCLUSION

The study proves the positive effects of PZ and PL *in vivo* and *in vitro*. PZ and PL both have shown hematoprotective effects on isolated erythrocytes. The study reveals that PL, the active constituent of PZ imparts its beneficial effects via activation of anti-oxidant enzymes. Our findings make it encouraging to study PL further as a potential agent against hematotoxic stress which is manifested as ADRs and encountered in response to various therapeutic regimes.

Acknowledgments

Authors appreciate Dr. Aamir Nazir, Scientist, Division of Toxicology, CSIR-CDRI, Lucknow for his valuable support in the writing and drafting of the manuscript. Dr.Sharad Sharma, Scientist, Division of Toxicology, CSIR-CDRI, Lucknow is acknowledged for his help in the processing of blood samples. This is CDRI Communication No. 9023.

Financial support and sponsorship

Author PS thanks to Department of Science and Technology, New Delhi for providing Inspire fellowship as scholarly grant (IF 10507). Authors PS and RKS appreciate the support from Director, CSIR-CDRI, Lucknow for providing necessary funds for the study.

Conflicts of interest

There are no conflicts of interest.

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